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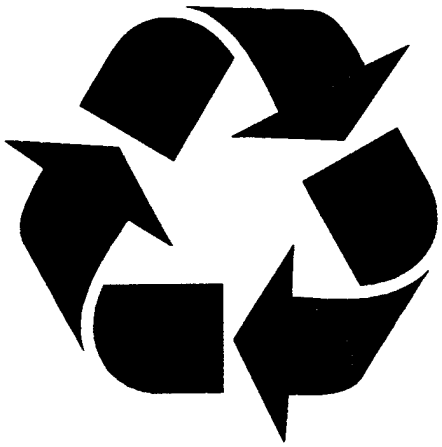
PROJECT SUMMARY:

BIOREMEDIATION OF CONTAMINATED SOILS USING THE WHITE ROT FUNGUS *Phanerochaete chrysosporium*

OUTLINE:



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1. Deliver final draft — Date: 17 April
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4. Graduate with M. E. in Environ Eng — Date: 5 May

BIOREMEDIATION OF CONTAMINATED SOILS USING

THE WHITE ROT FUNGUS

Phanerochaete chrysosporium

by

Michael S. Douglas

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ABSTRACT

Bioremediation is an enhancement of the natural biological oxidation processes that degrade organic matter. This is typically accomplished by introducing oxygen and nutrients to the soil that are necessary for the desired growth of naturally occurring bacteria. Fungal treatment is a specialized bioremediation process using fungi instead of bacteria to destroy contaminants. Recent interest in fungal treatment has grown due to the ability of "white rot" fungi to reduce a wide variety of organic and chlorinated organic compounds for which bacterial remediation methods have been ineffective. White rot fungi are wood-decay fungi that digest lignin in wood by the secretion of enzymes, giving wood a bleached appearance. Lignin is a complex polymer that gives structural support to woody plants. The ability to secrete extracellular enzymes enables the reduction of complex organic compounds outside the body of the fungi. Constituents of the lignin degrading system include lignin peroxidase, manganese peroxidase, veratryl alcohol, and hydrogen peroxide. In addition to lignin these fungi have been shown to degrade insecticides, herbicides, pentachlorophenol, creosote, coal tars, and heavy fuels. Research has shown that the fungi possess the ability to mineralize those contaminants to carbon dioxide, water, and basic elements. Use of white rot fungi in the treatment of contaminated soil augments the natural system by adding sufficient quantities of the fungal species. This technology can be applied in *ex situ* and *in situ* cases. Effective application requires adaptation of laboratory technologies to field conditions by carefully controlling temperature, oxygen, nitrogen level, pH, and moisture content. The development of bioreactors may help overcome some of these stumbling blocks.

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INTRODUCTION

Technologies for cleaning up hazardous wastes are often ineffective in handling complex mixtures of pollutants. Some of the most promising of the new technologies for solving hazardous waste problems involve the use of biological treatment, or "bioremediation," systems. Bioremediation systems use microorganisms, such as bacteria or fungi, for the degradation of toxic pollutants. They hold the promise of being an efficient and economical means of detoxifying contaminated water, soils and sediments, because they are much less disruptive than the commonly used options of excavation and incineration. Current developments have only begun to tap the potential of bioremediation systems, but they represent excellent alternative technologies for cleaning up hazardous wastes.

Decontamination of soils and sediments is one of the most difficult problems found at hazardous waste sites. Current technology does not adequately handle soil cleanup, usually because of the costs involved or the unsuitability of the technology to remove or destroy complex mixtures of pollutants. Furthermore, few microorganisms possess the ability to biodegrade toxic recalcitrant pollutants. Generally these compounds are poorly soluble in water, making them even less accessible to microbial attack [8]. For example, chlorinated aromatic compounds are highly toxic and resistant to degradation, thus they accumulate in the environment. Residual amounts of herbicide can persist for a significant period after application. However, certain fungi have been shown to mineralize complex aromatic compounds, like herbicides, and it is hoped their use may overcome many of the problems associated with bacterial degradation of these recalcitrant organopollutants.

This paper investigates the applicability of white rot fungi for the treatment of contaminated soils by evaluating available literary material. Primary sources include laboratory reports on the chemical and biological properties of the white rot fungus *Phanerochaete chrysosporium*, and current literature on the successful application of this fungus in field tests.

FUNGI DEFINED

"Fungi are eukaryotic, achlorophyllous, typically filamentous, spore-bearing organisms with the cell walls of most species consisting of chitin combined with other complex carbohydrates, sometimes including cellulose" [3]. In non-biologist's terms, they are multi-cellular, non-photosynthetic, with membrane-bound nuclei. There are currently over 120,000 species of fungi identified with 1500 new species being identified each year [2, 35, 55]. Mycologists tend to believe that fungi came from protozoan-like flagellate and, therefore, are not related to plants. A basic knowledge of what fungi are and how they metabolize nutrients is required to understand the fungal treatment process.

During the 19th century, all living matter was generally thought to be either plants or animals; however, the advent of the electron microscope during the past century has made it clear that the simple two kingdom approach is inadequate. Perhaps the most widely used classification system consists of three kingdoms based on functionality: Producers (plants), Consumers (animals), and Reducers (bacteria and fungi) [56]. The producers begin the food chain by taking up minerals from the earth and energy from the sun to generate new organic matter, a process called photosynthesis. The consumers can then use this organic matter as their source of energy and nourishment. As the producers and consumers die, the reducers decompose the organic matter back to basic minerals to complete the cycle. Fungi and bacteria are the principal reducers of organic matter [9].

Only during the past century has humankind begun to appreciate the beneficial uses of fungi as food, in food manufacturing and as antibiotics [33]. Mushrooms and truffles are used extensively as a food source, while yeast is relied upon in baking and brewing. Citric acid comes from fungi and is a prime ingredient in soft drinks. Roquefort cheese, penicillin and the vitamin riboflavin are all from different fungi. Drugs such as cortisone have been developed and fungi are used extensively in cancer research studies. Fungal biopulping and biobleaching are being developed to reduce the production of hazardous effluents in the paper industry. Fungal pathogens are being developed as natural insecticides, herbicides, and fungicides to reduce the use of hazardous chemicals. In addition, the prospect of using white rot fungi to destroy hazardous compounds in soils has generated a great deal of excitement in the past few years.

White rot fungi are wood-decay fungi that possess the ability to metabolize lignin [43]. Lignin is a naturally occurring, highly complex polymer that gives structural support to woody plants [1, 9]. It forms a matrix surrounding the cellulose, physically protecting it from microbial attack [5]. The white rot fungi seem to be unique in their ability to degrade lignin into carbon dioxide and water. This ability to degrade lignin imparts a selective advantage to white rot fungi because cellulose is unavailable as a carbohydrate source to non-lignin degraders [5].

CURRENT DEVELOPMENT

Phanerochaete chrysosporium is the most thoroughly investigated white rot fungus in the treatment of hazardous chemicals and wastes [33]. Interest in its use for bioremediation originated from research on the treatment of pulping and bleaching plant effluents. This research led to further studies that showed *P. chrysosporium* could degrade a broad range of organic and chlorinated organic compounds. Chemicals known to be degraded by white rot fungi include insecticides, herbicides, wood preserving chemicals, halogenated aromatic hydrocarbons, chlorinated aromatic compounds, dioxins, creosote, coal tars, and heavy fuels. These compounds are environmentally persistent because microorganisms are either unable to degrade them or do so very slowly.

Much of the initial research into the non-specific nature of *P. chrysosporium* was conducted by Dr. Steven Aust at Michigan State University in the early 1980's. In 1987, Dr. Aust left MSU to become the director of the Biotechnology Center at Utah State University. Dr. Aust co-founded Intech One-Eighty Corporation in Logan, Utah for the commercialization of fungal treatment technology in 1992. They have teamed up with EarthFax Engineering, Inc., who began field implementation of their technology in 1993 [1, 4, 8, 9, 15, 37, 55].

Richard T. Lamar of the USDA Forest Products Laboratory (FPL) has been working with John A. Glaser, manager for bioremediation at the EPA Risk Reduction Engineering Laboratory (RREL), since the mid 1980's to investigate the applicability of *P. chrysosporium* to the bioremediation of contaminated soils. These researchers are conducting full-scale field demonstrations of the fungal treatment technology to evaluate its suitability to field conditions [14, 19, 20, 30, 31, 32].

Another developer of fungal treatment technology is Tienzyme in State College, Pa. The founder, Ming Tien, is a biochemist in the department of molecular and cell biology at Pennsylvania State University. Their research has concentrated on developing superior strains of *P. chrysosporium* for bioremediation capabilities [10, 15, 49, 55].

Also, Mycotech Corporation in Butte, Montana is focusing on development and production of fungi for bioremediation. They culture and produce *P. chrysosporium* as a fungal powder. Their primary focus is on increasing yields to produce fungi in the quantities necessary for large scale field applications. Mycotech has joined with Groundwater Technology, Inc. to extend the use of fungi to large-scale remediation. In addition, Mycotech is looking at use of fungi as biopesticides [15].

GROWTH AND METABOLISM

Fungi may be either saprophytic, attacking dead organic matter and causing decay, or parasitic, attacking living tissue in plants and animals. The saprobes are reducers that play a significant role in the ecological cycle by recycling valuable nutrients back to the earth. The destruction of organic matter is directly proportional to fungal growth. In a system with unlimited nutrients, fungi have been shown to exhibit exponential growth with maximum rates of up to 0.5 h^{-1} in yeasts, and 0.36 h^{-1} reported in filamentous fungi [2]. Most bacteria metabolize nutrients inside their cell walls as illustrated in Figure 1. Because many complex organic compounds are insoluble, they are not easily transported into bacterial cells where *intracellular* enzymes would aid in their

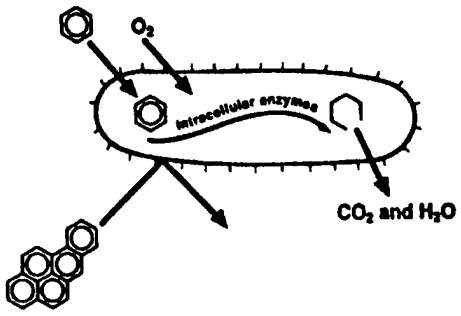

Figure 1. Bacteria metabolize nutrients intracellularly.

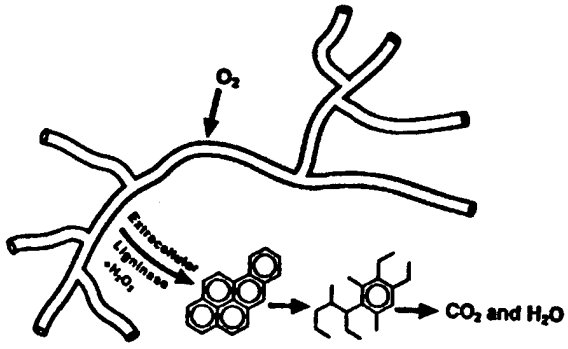

Figure 2. Fungi metabolize nutrients extracellularly.

consumption. Fungi, on the other hand, digest organic matter outside their body by secreting *extracellular* enzymes (Figure 2). These enzymes may include proteases, pectinases, lipases, cellulases, and ligninases [9]. This property of secreting enzymes enables the reduction of complex organic compounds outside the body of the fungi.

To completely harness the abilities of *P. chrysosporium*, it is necessary to understand its life cycle. This fungus can operate in two distinct metabolic cycles [20]. The primary, or growth, cycle is characterized by many structures formed during vegetative, sexual, and asexual reproductive phases [19]. The primary cycle consumes cellulose during the vegetative growth of mycelium. Eventually, the tissues of the mycelium form fruiting bodies that are shed, constituting the sexual phase of the primary cycle. Additionally, asexual reproduction occurs any time during the vegetative phase, and asexual spores are produced at all stages of the primary cycle [19].

The fungus uses cellulose as its primary growth substrate, but when large quantities of lignin are met the secondary metabolic cycle is entered [19]. During secondary metabolism the fungus secretes a complex mixture of lignin-degrading enzymes [5, 12, 20, 21, 26]. The first lignin-degrading enzyme, lignin peroxidase or "ligninase," from *P. chrysosporium* was described in 1983 [44]. These peroxidases have also been isolated from the cultures of other white rot fungi. Culture growths started with fungal spores generally do not become lignolytic until soon after about 4 days of growth [1].

The lignolytic system of *P. chrysosporium* is regulated by the availability of nutrients, oxygen, trace metals and pH, increasing the production of enzymes in the presence of various substrates, such as lignin and lignin model compounds [5]. These enzymes catalyze the oxidation of lignin by generating carbon-centered free radicals that result in cleavage of the lignin polymer. The resultant smaller-chained aromatic compounds are subsequently metabolized to carbon dioxide by more conventional enzyme systems [1, 9]. Important components of the lignin degrading system are lignin peroxidase (LiP), manganese peroxidase (MnP), veratryl alcohol (VA), hydrogen peroxide (H_2O_2), and a peroxide-generating enzyme, glyoxal oxidase [1, 5, 45, 48].

Lignin Peroxidase

The lignin degrading system of *P. chrysosporium* involves the excretion of lignin peroxidases (LiP). These enzymes are not very discriminating in what they will consume. The non-specific nature of the enzymes gives them the ability to oxidize non-lignin aromatics, resulting in compounds that the fungi can then mineralize. LiP production is triggered by nitrogen, carbon, or sulfur limitation. Since wood is nitrogen poor, wood inhabiting fungi naturally need less nitrogen. The low nitrogen levels in wood stimulate the production of ligninases, while at the same time limit the growth of competing organisms. Lignin, however, is an inadequate source of carbon and is degraded only in the presence of an additional carbon source [1].

Lignin peroxidases are able to oxidize substrates that are not normally oxidized by conventional peroxidases [5]. *P. chrysosporium* secretes a number of lignin peroxidases, over 10 of which have been detected in extracellular fluid of lignolytic cultures. Kirk *et al.* have named these proteins H1 to H10 [47]. H2 and H8 are the two major proteins most often discussed. The LiP isozyme H2 is predominant in nitrogen limited, agitated cultures, while LiP H8 is the predominant isozyme produced in nitrogen limited stationary mat cultures. The eventual use of LiP for industrial applications will depend upon the stability of these two proteins. LiP H2 is sufficiently stable under selected conditions of pH and temperature to suggest its use in waste treatment systems [52].

Manganese Peroxidase

While the reactions of LiP are the most thoroughly studied [12, 21, 22, 36, 41, 44, 48, 50, 51, 52, 54], the reactions of manganese peroxidase (MnP) have more recently come under review [21, 22, 28, 29, 38]. The manganese peroxidases have been most extensively characterized in *P. chrysosporium*. MnP catalyzes the H_2O_2 dependent oxidation of Mn2+ to Mn3+ [28]. This process is dependent on the presence of organic acids, also produced by the fungus. Some polycyclic aromatic hydrocarbons (PAHs) that are not LiP substrates are nevertheless degraded by white rot fungi due to a co-oxidative consequence of lipid peroxidation by MnP [38]. Findings of these studies suggest that MnP plays an important role in lignin biodegradation.

Veratryl Alcohol

Veratryl alcohol (VA) is a secondary metabolite of white rot fungi that is concurrently produced with peroxidases from glucose. VA stabilizes LiP and promotes the ability of this enzyme to oxidize a variety of physiological substrates. It has been well established that the addition of supplemental VA to cultures of *P. chrysosporium* causes an increase in lignin peroxidase activity in the extracellular fluid under nitrogen limitation [5].

Since VA plays diverse roles in white rot fungal metabolism, an extensive discussion over which role VA plays has been unleashed [26, 54]. Four roles have been proposed. First, addition of VA was thought to induce the production of LiP; however, recent research has negated this [10]. Second, VA acts as a stabilizer of lignin peroxidase activity by preventing H_2O_2-mediated inactivation of LiP [5, 26, 54]. This stabilizing effect is probably responsible for the observation that addition of VA enhances LiP levels. Third, it has been proposed that VA function as a charge-transfer mediator between the enzyme and a third substrate; however, this has not been generally accepted [54]. Lastly, VA functions as a substrate for H_2O_2-producing enzymes.

Hydrogen Peroxide

Hydrogen peroxide secretion appears about the same time that the fungus becomes lignolytic [1]. White rot fungi possess several systems for extracellular H_2O_2 generation, but the most common has been attributed to glyoxal oxidase [10, 11]. Hydrogen peroxide is synthesized in response to nitrogen starvation given a suitable carbon source. It is subsequently consumed in lignin degradation, indicating a peroxidative mechanism. H_2O_2 actually acts as a substrate that combines with LiP to generate carbon centered free radicals resulting in oxidation of the lignin. Since the fungus does not rely on lignin to produce LiP or H_2O_2, enzyme synthesis does not cease as the level of lignin is reduced, provided a nutrient deficient carbon source, such as cellulose, is present. This means that a lignin model compound may be reduced to essentially undetectable levels. However, at very low levels, the rate of degradation may be slow [1].

LABORATORY STUDIES

Despite considerable progress in understanding the model of lignin degradation by *P. chrysosporium*, relatively little is known about the details of degradation by the fungus. Further clarification of these metabolic pathways and the specific enzymes involved is necessary for the development of environmental cleanup strategies using the white rot fungus. Current research is focused on ways to optimize rates of biodegradation in order to apply this technology in waste treatment systems.

For most laboratory experiments involving *P. chrysosporium*, standard procedures for the growth of culture are used. The fungus is incubated at 37 to 39° C in nutrient nitrogen deficient liquid culture medium in 250-ml Wheaton bottles equipped with Teflon-sealed caps. This medium consists of 56mM glucose, 1.2 mM ammonium tartrate, mineral salts, and thiamine in 20 mM dimethylsuccinate (sodium) buffer (pH 4.5). The medium is inoculated with spores to establish cultures. During the first three days of incubation, cultures are grown under ambient atmosphere of air. After three days and at three day intervals thereafter, cultures are flushed with oxygen [5, 6].

Initial research by the Risk Reduction Engineering Laboratory assessed the effects of temperature, pH, and water potentials on the growth of the fungus in sterile and non-sterile soils [19]. *P. chrysosporium* is a mesophilic fungus whose optimal temperature for growth and lignin degradation is 39° C [4, 32, 34]. The fungus exhibits consistent growth between 25 and 39° C, while growth of the fungus decreases above 39° C. Soil temperatures under field conditions can be controlled by selecting the normal warm months for operation and by soil solarization. Soil water potential was shown to have a significant affect on the growth of the fungus in soil. The optimum soil moisture content is 30% [14]. As the soil water content decreases, fungal growth decreases. Also, since the fungus is an aerobe, its activity is influenced by the dissolved oxygen concentration. The use of pure oxygen rather than air results is much higher rates of lignin degradation [34].

DDT

DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) is a very powerful insecticide effective on contact, which has been banned from further use because of its alleged toxicity to birds. Several studies have been conducted using DDT as a model recalcitrant substrate [1,4, 15]. Laboratory research by Bumpus and Aust has demonstrated that DDT degradation by *P. chrysosporium* is promoted in response to nutrient nitrogen starvation. During a 30 day incubation period approximately 53% of the DDT was metabolized (Figure 3). Fortification of cultures with additional glucose at day 31 and day 61 demonstrated that additional degradation occurred under these conditions. The additional glucose may simply increase the overall rate of fungal metabolism, or allow the fungus to produce the required H_2O_2 as a co-product of the glucose oxidase system [6]. The rate of DDT disappearance appeared to decline as the concentration of glucose decreased. These results suggest that the ability to mineralize pollutants is dependent upon the availability of a carbon source that can serve as a growth substrate.

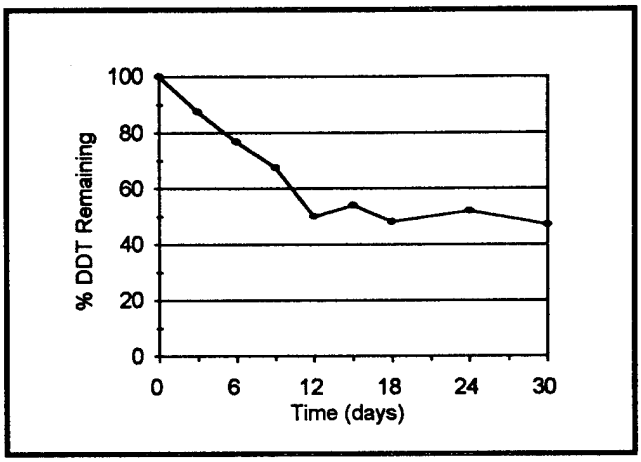

Figure 3. Degradation of DDT (adapted from Bumpus *et al.* [4]).

Degradation of DDT was demonstrated by DDT disappearance, metabolite identification, mass balance analysis, and [^{14}C]DDT mineralization studies [4]. Glucose utilization and fungal growth were measured by mycelium dry weight. During the first 3 days of incubation, vigorous growth occurred, achieving 50% of the fungal mass during this period. Although only 53% of the DDT was degraded during the 30-day incubation period, less that 1% of the DDT initially present still remained after 75 days of incubation [8].

The major metabolic pathway for DDT biodegradation in *P. chrysosporium* proceeds through a pathway in which DDT is first oxidized to 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol (dicofol), which is then dechlorinated to form 2,2-dichloro-1,1-bis(4-chlorophenyl)ethanol (FW-152) (Figure 4). Ultimately, oxidative carbon-carbon bond cleavage results in formation of DBP, which then presumably undergoes aromatic ring cleavage or reductive dechlorination followed by degradation to carbon dioxide [4].

It has been suggested that microorganisms could be used to remediate DDT-contaminated soils [4]. However, because substantial amounts of DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethene) will undoubtedly be present in sites contaminated with DDT, and because DDE is a metabolite of DDT in many microorganisms, it is necessary to identify microorganisms that can degrade DDE. The conversion of DDT to DDE has been termed 'a dead-end side reaction' because DDE is extremely resistant to microbial degradation and is regarded as a metabolic end product. However, a recent study by John Bumpus, *et al.*, demonstrated that DDE is degraded to CO_2 by *P. chrysosporium* as illustrated in Figure 4 [7].

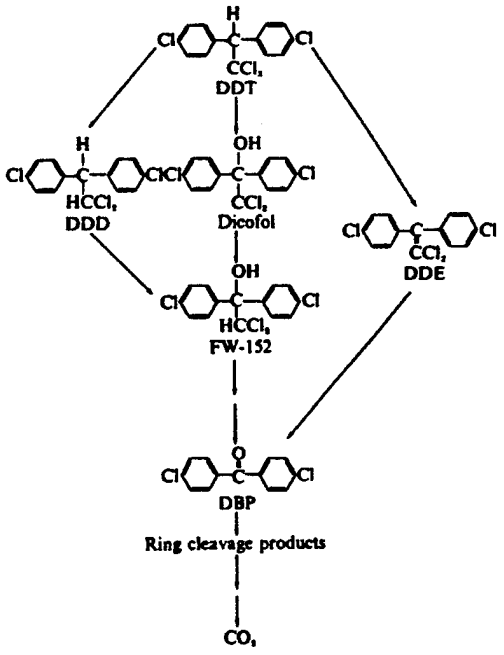

Figure 4. Proposed pathway of DDT and DDE.

PCP

Pentachlorophenol (PCP) is a common soil contaminant at wood preservation facilities where commercial formulations of PCPs were used, and is on the EPA's list of priority pollutants [31]. Researchers Aust and Bumpus conducted initial studies in 1988 on the ability of *P. chrysosporium* to mineralize PCP in nitrogen limited culture [37]. They presented evidence that the lignin degrading system of the fungus is responsible for extensive degradation of PCP. (Field studies by Lamar, *et al.*, later supported this evidence [30, 31].) Mineralization of PCP is promoted in nutrient nitrogen-limited cultures and suppressed in nitrogen-sufficient cultures. However, even under nutrient nitrogen-sufficient conditions, substantial mineralization of PCP occurred.

A prerequisite for any microorganism used in waste treatment systems is the ability to survive in the presence of the pollutant it is intended to degrade. This could pose problems for the fungus since PCP has also been used as a fungicide. Intrestingly, its success in the treatment of wood is due to its toxicity to fungi. Toxicity studies focused on the ability of *P. chrysosporium* to mineralize the pentachlorophenol in lethal concentrations. Initial results showed that PCP concentrations of 4 ppm (4 mg/liter) or higher prevented growth of the fungus and were lethal to the fungus when cultures were initiated with spores. However, it was found that if cultures were allowed to establish a mycelial mat for six days before the addition of PCP, the lethal effects of PCP could be circumvented. This suggests that conditions may be developed which will allow this fungus to grow and degrade pollutants in a number of potentially toxic environments.

Effect of PCP on growth of *P. chrysosporium*

PCP concentration (mg/liter)	Mean amount of glucose converted to CO_2 (nmol)
0	31.6
1	42.6
10	28.1
100	25.9
500	0.5

The table above shows that increasing initial PCP concentration up to as high as 100 mg/liter had little ability to inhibit fungal growth. For this toxicity test, PCP and glucose were added to 6-day-old nitrogen-limited cultures which were then incubated for an additional 24 days [37].

Crystal Violet

Crystal violet (N,N,N',N',N",N"-hexamethylpararosaniline) has seen extensive use in human and veterinary medicine, as a biological stain, and as a textile dye. Some success in crystal violet decolorization in wastewater has been reported; however, wastewater treatment facilities are often unable to completely remove dyes from contaminated wastewater. The inability of most bacteria to degrade crystal violet has been attributed to the fact that this dye is toxic to many microorganisms. In a study conducted by Bumpus *et al.* [5], the crystal violet dye underwent extensive degradation when added to nitrogen-limited lignolytic culture of *P. chrysosporium*. It was demonstrated that the first steps of crystal violet degradation by *P. chrysosporium* are extracellular. It was also found that supplementing the system with additional glucose (56mM) promoted biodegradation.

TNT

TNT (2,4,5-trinitrotoluene) is a significant soil contaminant at many sites that produce the explosive. The compound is resistant to natural bacterial degradation; however, lignolytic cultures of *P. chrysosporium* have been shown to mineralize 40% of TNT in as little as 3 days [1, 32]. Figure 5 shows that non-lignolytic cultures mineralized only about 3% in the first 3 days; however, upon becoming lignolytic the cultures then mineralized

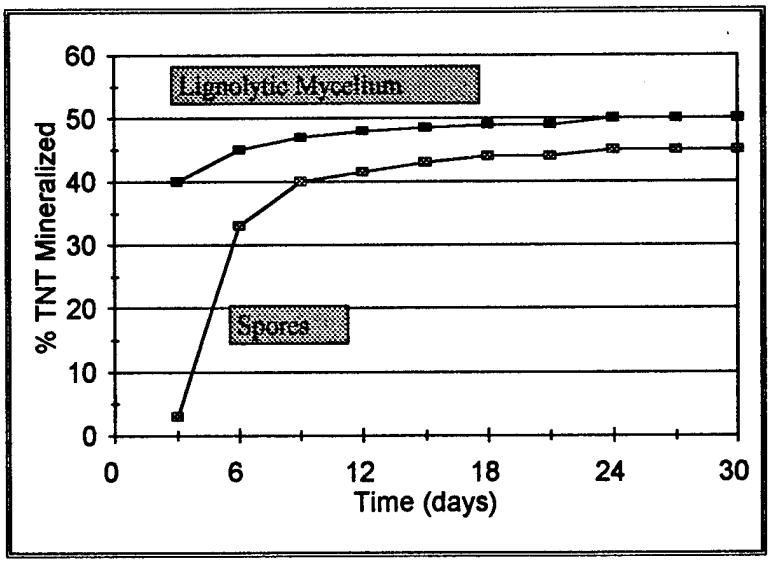

Figure 5. TNT degradation by *P. chrysosporium* started with lignolytic mycelium or spores.

about 30% of the TNT in the next 3 day period [1]. A readily utilized carbon source is necessary for mineralization; therefore, addition of glucose frequently results in another phase of mineralization [1]. Studies by Michels *et al.* have demonstrated that only lignolytic cultures of *P. chrysosporium* mineralize TNT at an appreciable rate, indicating that the lignolytic system is involved in the reactions of TNT degradation [36].

Herbicides [2,4-D and Atrazine]

The aromatic compound 2,4-D (2,4-dichlorophenoxyacetic acid) is widely used as a systemic herbicide for controlling broadleaf vegetation. It is usually applied as a post-emergence herbicide. 2,4-D works by causing the plant to grow too fast, changing growth patterns and resulting in death of the plant. The large scale use of chlorophenols has resulted in the classification of chlorophenols as priority pollutants. Laboratory studies by Khadar Valli and Michael Gold in 1990 demonstrated the ability of *P. chrysosporium* to mineralize 2,4-D under secondary metabolic conditions [53]. Atrazine is another chlorinated aromatic herbicide commonly used in both agriculture and forestry for vegetation control [13]. One study in 1993 was unable to demonstrate the mineralization of atrazine by *P. chrysosporium* [13]; however, another study later that year demonstrated partial conversion of the herbicide [40] by the fungus.

BIOREACTORS

Fungi have not traditionally been used in waste treatment systems because many of them are pathogenic. Even the benign forms disturb the normal processing of waste materials, and fungi are regarded as nuisances since they can cause the precipitation of the sludge blanket in activated sludge systems[20]. However, the unique lignin degrading capabilities of the white rot fungus *P. chrysosporium* offer significant enough advantages that its role in biological reactors should be reconsidered.

Because of its broad biodegradative abilities, Bumpus and Aust have proposed that it may be possible to develop waste treatment systems based on the use of this microorganism. Treatment requires sufficient quantities of the fungal species, controlled temperature, oxygen and nitrogen level, pH, and moisture content. The organisms must first grow to a sufficient biomass before being switched to a nitrogen deficient state that causes the desired enzymes

to be produced. These requirements make practical applications in the field more challenging. The development of bioreactors may overcome some of these stumbling blocks and allow simulation of laboratory experiments on a large scale in the field.

In traditional liquid reactors, toxic and hazardous pollutants in liquid form are brought into contact with microorganisms to accelerate the degradation process. The stirred reactor design is extremely popular in industry and easy to operate. The mixing and aeration that occurs insure that sufficient oxygen is present for the greatest possible oxidations of the organic compounds. The main problem with these reactors in fungal growth is sensitivity to shear.

At the New Jersey Institute of Technology, Newark, researchers have been conducting laboratory studies to determine kinetic rate reaction constants for continuous flow reactors and have published initial reactor design parameters for fungal treatment [16, 34]. In their study, they found that biodegradation rate improved by a factor of 40 when the fungus was immobilized. Using a packed-bed reactor (Figure 6) with silica-based support

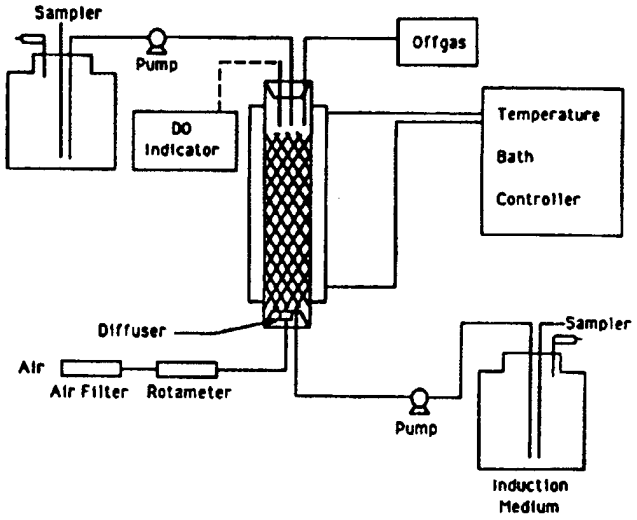

Figure 6. Packed bed Reactor [34].

to immobilize the fungus, growth of the fungus was induced for five to seven days. After the initial growth period, the enzyme ligninase was produced in the fungi by subjecting the reactors to nitrogen starved conditions. Steady state of degradation was usually reached five days after toxics were introduced with flow rates of 0.4 ml/min. Growth rates experienced for the packed-bed reactor were 0.19 h^{-1}. This values of the rate constant for the packed-bed reactor clearly indicates the importance of immobilizing the fungus on a support.

Combinations of a variety of different treatment technologies should be considered in conjunction with bioremediation. A three-phased sequential treatment system, for example, is being developed and is currently being evaluated in bench studies [42]. The approach integrates soil washing technology (Phase I) with dewatering and chemical filtration (Phase II). These first two phases combine to reduce the volume of material to be treated from 100 to 100,000 fold. This volume reduction concentrates the contaminant and facilitates biodegradation of the pollutants (Phase III). Figure 7 illustrates the process flow for these three phases. Specially patented bacteria

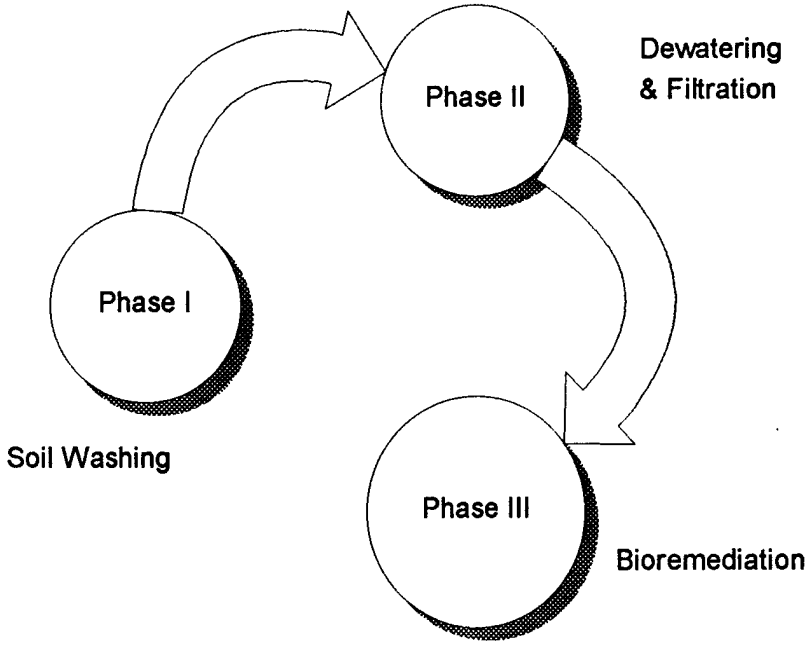

Figure 7. Sequential Treatment.

housed in continuous flow bioreactors are being used in the current study. However, white rot fungi have potential for this type application since they also can use high molecular weight creosote constituents as growth substrates. This process is designed to treat contaminated soil, groundwater, and surface water simultaneously. Other advantages include the ability to remove toxic compounds and control the environmental conditions in the biological treatment phase. Combining processes to overcome traditional barriers may make fungal remediation applicable to a wide range of hazardous waste problems.

FIELD APPLICATION

Many new companies are cropping up all over the United States for the development of fungal treatment applications in the field. *P. chrysosporium* has the potential for bioremediation of soils because of its ability to thoroughly degrade a wide variety of structurally diverse organopollutants, including insecticides, herbicides, fungicides, explosives, PCBs, and heavy oils [1, 4, 5, 7, 9, 15, 23, 27, 30, 32, 37, 47]. The fact that these compounds are reduced to carbon dioxide is important because it demonstrates that a metabolic pathway exists for the complete biodegradation of each of these compounds. It also implies that intermediates formed during biodegradation of the parent compound are metabolized. Some of the more common substructures of lignin resemble the chemical structure of many persistent organic compounds contaminating the environment. This structural similarity has given sufficient reason to pursue application of white rot fungi to the bioremediation of soils contaminated with hazardous waste constituents [6].

Biotreatment processes used in laboratories and treatment plants must be adapted to the field. Engineers are using the principles developed in the laboratory to design systems that will work in the field. To help accelerate the development, demonstration, and use of new treatment technologies, the EPA established the Superfund Innovative Technology Evaluation (SITE) program in 1986. SITE is a public-private partnership where the costs and monetary risks are shared. Often it is necessary to use these technologies in combination with each other to deal with the mixtures of chemical substances present. The complex mixture of pollutants at industrial hazardous waste sites makes cleaning up contaminated soils challenging. The first step in field testing a biological treatment

process is to characterize the site, its contaminants, and the various environmental factors that will enable the growth of the fungus.

Soil is not the natural habitat of the fungus; however, recent studies have shown that growth of *P. chrysosporium* within the soil can be accomplished with larger quantities of inoculum [32]. Researchers Steven Aust, John Bumpus, and Tudor Fernando have examined the use of a number of inexpensive and readily available carbon sources for their ability to support growth of *P. chrysosporium* [17]. Common items such as wood chips and corn cobs can serve as a bulking agent and as a carbon source for the fungus [6]. These materials are an ideal nutrient source since they are natural substrates for the organism and are naturally low in nutrient nitrogen yet contain the necessary nutrients for growth and a carbon source. The Clyde Engineering Service in New Orleans is experimenting with damp corrugated cardboard boxes as the growth medium for the fungus. Their hope is that *in situ* treatment using ordinary cardboard will eliminate the requirement for tilling the soil, because the corrugation in the cardboard would provide an aerobic environment for the growth of the fungus [15]. Other studies have shown that growth was enhanced when peat moss was used included as a source of organic carbon for fungal growth [30].

Contaminated soil has traditionally been hauled to a disposal site and replaced with clean backfill. As landfills become increasingly scarce, the cost of soil disposal has risen sharply, making innovative biological treatment a cost-effective alternative. Furthermore, biological treatment results in the destruction of the contamination rather than the transfer of chemicals to another media. Bioremediation of contaminated soils generally uses one of two basic techniques: biostimulation or bioaugmentation [39]. Biostimulation uses microbes that are already found in the soil by stimulating their growth through the introduction of nutrients. Bioaugmentation involves culturing the microorganisms independently and adding them to the site. The selected microorganisms must be carefully matched to the waste contamination present in the soil, and must favorably compete with the native microbes found in the soil. Fungal remediation of soils is a bioaugmentation process because white rot fungus is not native to soil.

Bioremediation of contaminated soil may involve excavation of the soil and construction of a lined treatment cell. This "*ex situ*" treatment has some distinct advantages. It allows for better control of temperature, nutrient concentration, moisture content, and oxygen availability. A substrate inoculated with the fungus is easily mixed with the contaminated soil when it has been excavated. Also, the presence of a liner prevents the migration of the contaminants, and it is easy to demonstrate that the site is clean when the soil is excavated. The moisture content of the soil must be maintained at an optimal level for growth. To accomplish this, a carefully engineered soil mound is constructed containing a network of perforated pipe for forcing air through the soil. A second network of pipes is provided for water delivery to add moisture to the soil. The mound is usually constructed on a pad and under-drained to collect runoff. The fungi grow until the growth substrates are fully consumed, degrading the hazardous compounds in a period of 4 to 12 weeks depending on soil conditions and initial contaminant concentration. The direct costs associated with *ex situ* soil treatment using white rot fungi have been estimated at $200 to 400 per ton of contaminated soil [15]. Figure 8 shows a cross-sectional view of a typical soil treatment mound.

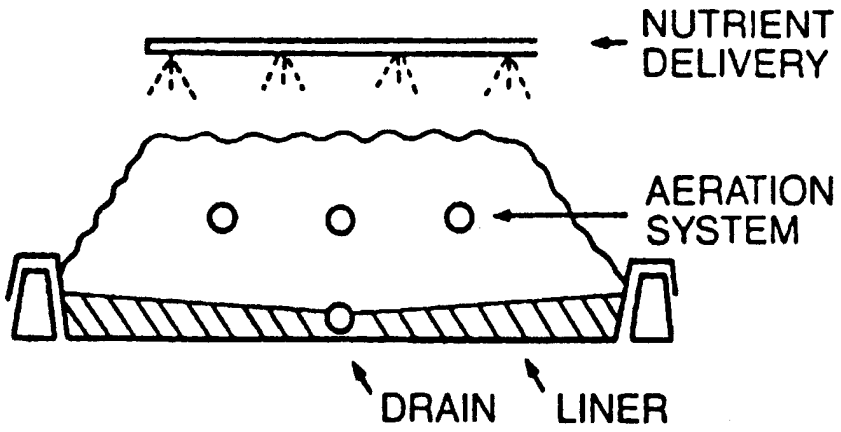

Figure 8. Typical *ex situ* treatment process [18]

If excavation is impractical, the treatment may be conducted without disturbing the contaminated site by using a recirculating injection well system. This process is considered "*in situ*" treatment. Biologically cleaning up soils *in situ* is much more effective and inexpensive than excavating the soils before treatment [39]. When saturated soils are contaminated with petroleum hydrocarbons, most of the constituents of petroleum products are relatively insoluble. This means a major portion of the contaminant will be trapped between soil particles or adsorbed to the soil. Biodegradation in place is, therefore, a faster and less costly method than the traditional "pump and treat" approach for the treatment of contaminated soil below the water table. *In situ* treatment generally involves establishing a hydrostatic gradient through the area of contamination, and can be carried out for saturated soils or unsaturated soils [1, 6, 32, 57]. The same principles are involved, but the methods of delivering the oxygen and mineral nutrients differ [18]. The delivery of the nutrients is accomplished by dissolving them in groundwater that is recirculated through the contaminated area. Groundwater is withdrawn from wells located down-gradient of the contaminant, amended, and reinjected up-gradient using wells or trenches (Figure 9).

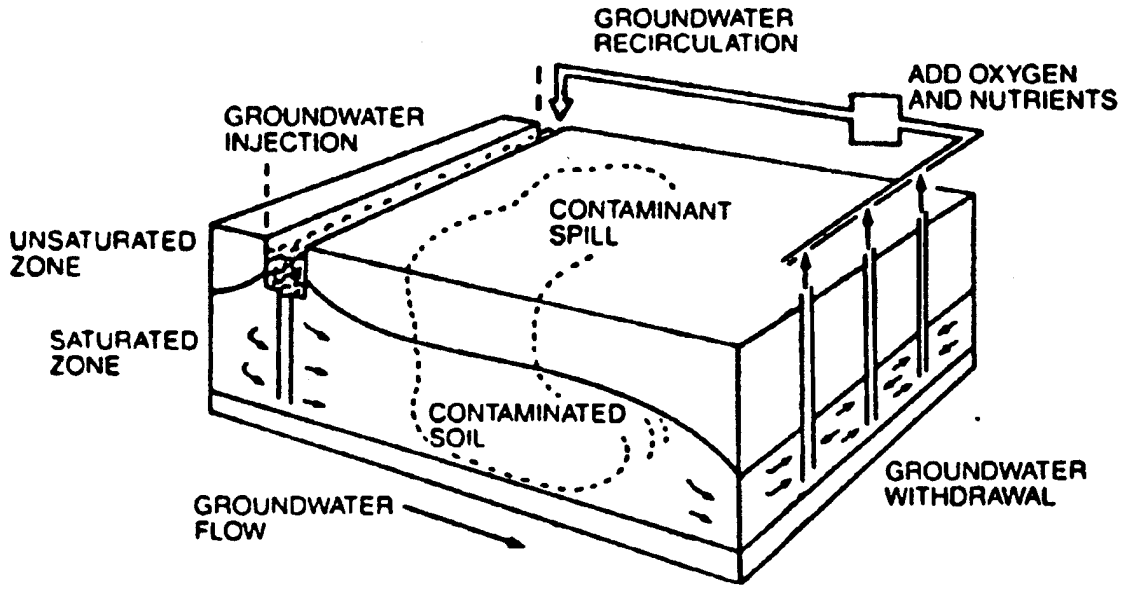

Figure 9. Typical *in situ* treatment process [18]

At the Brookhaven Wood Preserving facility in Brookhaven, MS, the RREL and the FPL conducted a study in 1992 to determine the use of white rot fungi to destroy PCP and polynuclear aromatic hydrocarbons (PAH's found in creosote) in contaminated soil surrounding the wood treatment facility [14]. The study was conducted using the species *P. chrysosporium* and *Phanerochaete sordida* because they exhibit the best growth potential in soil containing wood preserving wastes. The objective of the treatability study was to determine applicable fungal species, inoculum loading level, treatment time, and the need for nutrient supplements. The study was conducted under optimum growth conditions with temperatures greater than 80°F and soil moisture content greater than 30%. Initial findings show that levels of PCP and PAHs found in the leachate and in the air samples were insignificant, indicating low leachability and low potential of air borne contaminant transport as a result of the fungal activities.

The ability of white rot fungi to deplete PCP from soil was examined in another field study by the FPL in 1990 at the site of a former tank farm in Oshkosh, Wisconsin [30, 31]. There were three 15,000 gallon tanks on the storage area, which were reportedly used to store a wood preservative composed of mineral spirits and PCP. The tanks were situated on a gravel bed that overlaid the contaminated soil. After the gravel was removed, the soil was

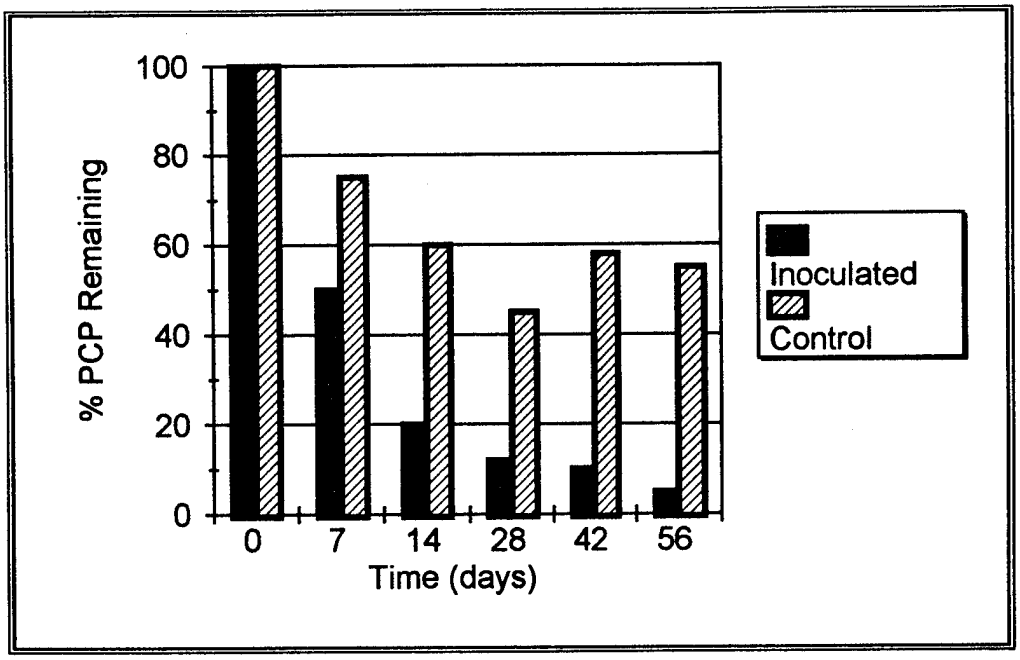

Figure 10. Reduction of Pentachlorophenol in soil [30,31]

physically mixed *in situ* to a depth of 30 cm with a rototiller to allow volatilization of mineral spirits that were also present in the soil. (Preliminary experiments demonstrated that the mineral spirits completely prevented the growth of the fungus.) Inoculation of soil containing 250 to 400 μg of PCP per gram of soil resulted in dramatic decreases (51 to 77%) in the PCP between days 1 and 22, with an overall decrease of 88 to 91% of PCP in the soil in 45 days (Figure 10). Inoculum consisted of aspen chips that were thoroughly grown through with *P. chrysosporium*. Reduction of PCP in control samples was attributed to absorption into the wood chips. When the losses of PCP through mineralization and volatilization were negligible, most of the PCP was converted to nonextractable soil-bound products.

It is evident that the development of field soil treatment technology based on *P. chrysosporium* has become the focus of intense research. Many other bench- and full-scale treatment studies using the white rot fungus *P. chrysosporium* are currently being conducted by companies such as Mycotech and Groundwater Technology [15]. While the results of most of these tests are unpublished at the time of this writing, the committed effort indicates a strong belief that this fungus can be a cost effective alternative in the remediation of contaminated soils.

CONCLUSIONS

The use of microorganisms for bioremediation of contaminated soils is an attractive concept. Biological treatment results in the destruction of the contamination rather than the transfer of chemicals to another media. In numerous cases it has been shown to be effective, economical and environmentally compatible. Processes utilizing typical bacteria have shown limited success for some of the more recalcitrant and toxic pollutants. However, results indicate that the use of white rot fungi to degrade these pollutants in contaminated soils has tremendous potential. Low solubility and high toxicity of many of these pollutants are responsible for the their recalcitrance, because degradation by traditional intracellular enzymes is precluded. The ability to secrete extracellular enzymes is, therefore, the single most important factor in the success of the fungi to reduce complex organic compounds. Additionally, the non-specific nature of the enzymes gives them the ability to oxidize non-lignin aromatics.

Comparison of the laboratory and field studies suggests that rates of depletion in field soils could be greatly enhanced by regulating environmental conditions to favor both optimal fungal growth and lignolytic activity. Ligninase is not produced during the initial spore phase, so conditions must first be favorable for the primary growth of the fungus to establish a mycelial mat. This is also necessary to overcome the toxicity of some pollutants. Subsequently, a nutrient deficient condition must be induced for the generation of the extracellular enzymes needed in the degradative process. The development of bioreactors may help overcome some of these stumbling blocks.

Since the growth of white rot fungi has been demonstrated in both solid and liquid phases, it is feasible the they could be used *in situ* in saturated soils. However, nutrient conditions will be much easier to control with the more traditional pump and treat methods. Once the contaminated groundwater is extracted, it can be cycled through a liquid phase bioreactor. The stirred tank design is extremely popular in industry and easy to operate. The mixing and aeration that occurs insure that sufficient oxygen is present for the greatest possible oxidations of the organic compounds. The main problem with these reactors in fungal growth is sensitivity to shear. Immobilization of the fungus in a support material to protect it from shear, while allowing mixing and aeration, make the Rotating Biological Contactor (RBC) an attractive reactor design. Once the mycelial mat is established on the discs, the use of additives such as glucose could provide the necessary nitrogen deficient carbon source for the generation of the enzymes. Alternatively, the use of ligninases as additives may prove to be a viable option in some bioreactors because it would not require the presence of the fungus itself. The ligninase would initiate the ring cleavage of complex organic compounds so that other naturally occurring microorganism could further degrade them.

For treatment of unsaturated soils, *ex situ* methods have some distinct advantages. They allow for better control of temperature, nutrient concentration, moisture content, and oxygen availability. A substrate inoculated with the fungus as well as additives can be easily mixed with the contaminated soil when it has been excavated. However, widespread use in the treatment of soils will require a complete understanding of the stability of the soil-bound transformation products.

GLOSSARY

Atrazine	Herbicide, (2-chloro-4-ethylamino-6-isopropylamino-s-triazine)
Crystal violet	Textile dye, (N,N,N',N',N",N"-hexamethylpararosaniline)
DDE	Metabolite of DDT, (1,1-dichloro-2,2-bis(4-chlorophenyl)ethene)
DDT	Insecticide, (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane)
FPL	USDA Forest Products Laboratory
H_2O_2	Hydrogen peroxide
LiP	Lignin peroxidase, or ligninase
MnP	Manganese peroxidase
P.chrysosporium	The white rot fungus, *Phanerochaete chrysosporium*
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls, used as insulating oil and lubricant
PCP	Pentachlorophenol, wood preserving compound and fungicide
RREL	EPA Risk Reduction Engineering Laboratory
SITE	Superfund Innovative Technology Evaluation program
TNT	Explosive, (2,4,6-trinitrotoluene)
2,4-D	Systemic post-emergence herbicide, (2,4-dichlorophenoxyacetic acid)
VA	Veratryl alcohol

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